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Increasing the synthetic performance of penicillin acylase PAS2 by structure-inspired semi-random mutagenesis

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A semi-random mutagenesis approach was followed to increase the performance of penicillin acylase PAS2 in the kinetically controlled synthesis of ampicillin from 6-aminopenicillanic acid (6-APA) and activated D-phenylglycine derivatives. We directed changes in amino acid residues to positions close to the active site that are expected to affect the catalytic performance of penicillin acylase: αR160, αF161 and βF24. From the resulting triple mutant gene bank, six improved PAS2 mutants were recovered by screening only 700 active mutants with an HPLC-based screening method. A detailed kinetic analysis of the three most promising mutants, T23, TM33 and TM38, is presented. These mutants allowed the accumulation of ampicillin at 4–5 times higher concentrations than the wild-type enzyme, using D-phenylglycine methyl ester as the acyl donor. At the same time, the loss of activated acyl donor due to the competitive hydrolytic side reactions could be reduced to <20% with the mutant enzymes compared to >80% when using wild-type PAS2. Although catalytic activity dropped by a factor of 5–10, the enhanced synthetic performance of the recovered penicillin acylase variants makes them interesting biocatalysts for the production of β-lactam antibiotics.

Keywords: β-lactam antibiotics/penicillin acylase/semi-random mutagenesis/synthesis

Introduction

For several decades, penicillin acylase (penicillin amidase, PA, EC 3.5.1.11) has been used for the hydrolytic release of 6-aminopenicillanic acid (6-APA) from fermentatively obtained natural penicillins, which is a central step in the production of semi-synthetic β-lactam antibiotics. Recently, also biocatalytic processes using this enzyme for the kinetically controlled condensation of activated acyl moieties with 6-APA or other β-lactam nuclei have been developed (Bruggink *et al*., 1998). Although competitive with traditional chemical synthesis routes, these enzymatic procedures suffer from the fact that the desired synthesis of β-lactam antibiotics is accompanied by two side reactions, leading to the loss of activated acyl donor and limiting the product yield: (1) hydrolysis of the activated acyl donor and (2) hydrolysis of the formed antibiotic. Efforts to overcome this problem have mostly focused on a change in reaction conditions, such as pH optimization (Youshko *et al*., 2002b), the use of supersaturated substrate solutions (Youshko *et al*., 2004) or immobilized enzyme preparations (Alvaro *et al*., 1990) and medium engineering (Fernández-Lafuente *et al*., 1996). However, it was shown that the intrinsic kinetic properties of the biocatalyst employed also have a major impact on the efficiency of the synthetic reaction (Hernández-Jústiz *et al*., 1999; Youshko *et al*., 2002a; Alkema *et al*., 2003).

During PA-catalyzed antibiotic synthesis, the formation of the synthetic product Ps, i.e. the antibiotic, and also the formation of the hydrolytic byproduct Ph proceed via the formation of a covalent acyl-enzyme intermediate EAc, which is the rate-limiting step in the catalytic process (Figure 1). EAc can then be attacked by one of two possible nucleophiles: either the β-lactam nucleus N, leading to the desired product Ps, or by a water molecule, which results in the formation of Ph. The ratio between the initial production rates of synthetic product (v_{Ps}) and hydrolytic product (v_{Ph}) and the maximum level of product accumulation that is obtained (Ps_{max}) depend on the initial concentrations of β-lactam nucleophile N and acyl donor AD and the kinetic parameters of the enzyme. In fact, antibiotic formation in PA-catalyzed reactions is sufficiently described by only three complex kinetic parameters, α, β_0 and γ, according to Equation 1 (Youshko *et al*., 2002a):

$$\frac{d[Ps]}{d[Ph]} = \frac{\beta_0[N][AD] - \alpha[Ps](1 + \beta_0\gamma[N])}{(1 + \beta_0\gamma[N])([AD] + \alpha[Ps])} \quad (1)$$

with $[AD]_0 = [AD] + [Ps] + [Ph]$ and $[N]_0 = [N] + [Ps]$.

Experimentally, parameters β_0 and γ can be directly determined, since they relate the initial rates of product and byproduct formation to the concentration of nucleophilic β-lactam compound in a Michaelis–Menten-type equation:

$$\left(\frac{v_{Ps}}{v_{Ph}}\right)_{ini} = \frac{1}{\gamma} \cdot \frac{[N]}{\frac{1}{\beta_0\gamma} + [N]} \quad (2)$$

where $1/\gamma$ is the maximum that is reached for $[N] \rightarrow \infty$ and β_0 is the initial slope of a $(v_{Ps}/v_{Ph})_{ini}$ vs. [N] plot. The third

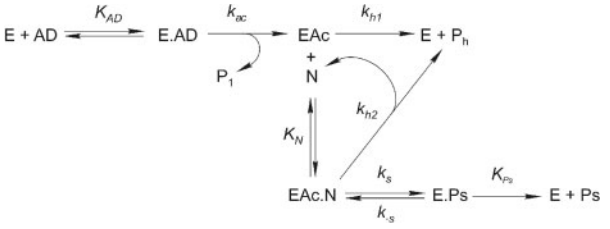

Fig. 1. Kinetic scheme of the synthesis and hydrolysis reactions catalyzed by penicillin acylase via the formation of a covalent acyl-enzyme intermediate EAc. Designations: E, free enzyme; AD, activated acyl donor; E.AD, noncovalent enzyme–acyl donor complex; P_1, first reaction product that is released upon acylation of the enzyme; Ph, product of hydrolysis reactions; N, β-lactam nucleophile; EAc.N, covalent acyl-enzyme intermediate with noncovalently bound nucleophile; Ps, product of the synthesis reaction, i.e. the antibiotic; E.Ps, enzyme–antibiotic complex. Rate constants k_{ac}, k_{h1}, k_{h2}, k_s and k_{-s} refer to different reaction steps as indicated in the scheme. K_{AD}, K_{Ps} and K_N are the binding constants of AD and Ps to the free enzyme and of N to the acyl-enzyme intermediate, respectively.

parameter, α, introduces the fact that the synthesized antibiotic is also a substrate for the enzyme, leading to its hydrolysis:

$$\alpha = \frac{\left(\frac{k_{cat}}{K_m}\right)_{PS}}{\left(\frac{k_{cat}}{K_m}\right)_{AD}} \quad (3)$$

The parameter α actually reflects the relative specificity of PA for the antibiotic with respect to the activated acyl donor and should be as small as possible to allow high product accumulation in a synthesis reaction. The parameters β_0 and $1/\gamma$, in contrast, preferably should be high to prevent the formation of large amounts of hydrolytic byproduct. The use of a PA with favorable kinetic parameters for the synthesis of a specific antibiotic thus takes a key position in the design of an efficient production process.

Biocatalysts with desired properties can be obtained either from nature, applying search strategies that range from traditional screening of culture collections to screening of metagenomic gene banks (Handelsman *et al*., 1998; Lorenz *et al*., 2002) or by improving already available enzymes (Kazlauskas, 2000; Zhao *et al*., 2002). Examples for the first approach are the PAs of *Kluyvera citrophila*, *Bacillus megaterium*, *Proteus rettgeri* and *Alcaligenes faecalis*, which were all detected for the first time by assaying pure bacterial cultures (Huber *et al*., 1972; Barbero *et al*., 1986). Unfortunately, none of these enzymes seems to be better suited for synthetic purposes than the already known PA of *Escherichia coli* (Hernández-Justiz *et al*., 1999). In contrast, an enzyme with better kinetic properties for 6-APA-derived antibiotic production, PAS2, was isolated from a soil metagenomic gene library (Gabor *et al*., 2004a). PAS2 allows significantly improved antibiotic yields and, concomitantly, lower amounts of hydrolytic byproduct than the industrially employed *E.coli* PA with which it has 51.4% of its amino acid residues in common (Gabor *et al*., 2004b). Like the *E.coli* enzyme, PAS2 is a periplasmatic enzyme that consists of an α-subunit (25.5 kDa) and a β-subunit (61.9 kDa).

In this study, we explored the possibility of further increasing the synthetic performance of PAS2 for ampicillin production, using a semi-random mutagenesis procedure. By simultaneous randomization of three amino acid residues close to the active site, a gene library comprising 6000 original mutants was created. Since the three selected residues are known to affect greatly the kinetic properties of *E.coli* PA (Alkema *et al*., 2000, 2002a), we anticipated that the mutants would show significant variations in catalytic behavior. Indeed, six clearly improved enzymes were found on screening only 700 active mutants. A detailed kinetic analysis of the three most promising PAS2 derivatives is presented.

Materials and methods

Chemicals

Ampicillin and cephalexin were purchased from Sigma. D-Phenylglycine (PG) was obtained from Acros. 6-Aminopenicillanic acid (6-APA), 7-aminodesacetoxycephalosporanic acid (7-ADCA), D-phenylglycine methyl ester (PGM) and D-phenylglycinamide (PGA) were provided by DSM Life Sciences (Delft, The Netherlands). The colorimetric substrate 2-nitro-5-[(phenylacetyl)amino]benzoic acid (NIPAB) was synthesized according to the following procedure: 5.08 g of 5-amino-2-nitrobenzoic acid were dissolved in 200 ml of dry acetone, to which 4 ml of phenylacetyl chloride were added slowly. A solid precipitate formed after 30 min and the mixture was stirred at room temperature overnight. After filtration, the yellow acetone solution was concentrated to 50 ml and added dropwise to 250 ml of vigorously stirred water. The product precipitated as a slightly yellow solid, which was recrystallized from water to give a white solid (3.80 g yield, i.e. 91% based on the fact that only 50% of the amine reacts and the other half acts as a base to neutralize the liberated HCl). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 3.50 (s, 1H, COOH); 3.65 (s, 2H, CH_2); 7.19–7.27 (m, 5H, Ph); 7.79 (d, 1H, Ph); 7.95 (m, 2H, Ph); 10.77 (s, 1H, NH). Phenylacetyl-L-leucine (PAL) was obtained through standard organic chemical peptide coupling chemistry: 6.55 g of L-leucine were dissolved in 50 ml of 2 M NaOH. The solution was cooled to <10°C, then 8.5 g of phenylacetyl chloride were added dropwise, keeping the temperature <10°C and the pH >10. After complete addition, the mixture was allowed to warm up to room temperature and was stirred for a further 1 h. The water was acidified to pH 4 with concentrated phosphoric acid to precipitate the product. Filtration and washing with ample amounts of water to remove traces of phenylacetic acid gave the product, which was recrystallized from hot water–ethanol. Drying under vacuum yielded 7.1 g of PAL (57%), m.p. 131–132°C (uncorrected). HPLC analysis showed that there was <0.7% phenylacetic acid in the sample. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.90 (dd, 6H, CH_3); 1.40–1.65 (m, 3H, $CHCH_2$); 3.42 (s, 2H, CH_2); 4.19 (m, 1H, Cα); 7.18–7.35 (m, 5H, Ph); 8.35 (d, 1H, NH). ^{13}C NMR (300 MHz, DMSO-d_6), δ (ppm): 21.3 (CH_3); 22.9 (CH_3); 24.4 (CH); 42.0 (CH_2); 50.3 (Cα); 126.3 (CH); 128.2 (CH); 129.0 (CH); 136.4 (Cq); 170.2 (CO); 174.2 (CO).

Plasmids and strains

For all plasmid constructs described in this study, *E.coli* TOP 10 [Δ(Ara–Leu)7697, Invitrogen] was used as the host organism. pBADPAS2 is a 6.7 kb plasmid carrying the wild-type *pas2* gene under the control of the pBAD promoter (Gabor *et al*., 2004b). pPAS2CAT is a similar construct, just conferring chloramphenicol instead of ampicillin resistance to prevent interference of β-lactamase activity with β-lactam antibiotic synthesis experiments in whole cells. To obtain this construct, pBADPAS2 was cut with *Bsp*HI (New England Biolabs) to remove the β-lactamase gene. The remaining 5.7 kb fragment was isolated from gel with a QIAEX II gel extraction kit (Qiagen), blunted with Klenow polymerase (Invitrogen) and ligated to an also gel-purified blunt 1.2 kb fragment with T4 ligase (Invitrogen). The 1.2 kb fragment carried a chloramphenicol acetyltransferase gene (*cat*) and originated from the plasmid pEC (provided by DSM, Delft, The Netherlands) (Alkema *et al*., 2000), from which it was removed by *Bsp*HI/*Eco*RI restriction (New England Biolabs). All DNA manipulations were carried out according to the instructions of the manufacturers of the materials used. The construct was transferred to *E.coli* by electroporation and confirmed by restriction analysis. All media used to grow clones carrying the wild-type pPAS2CAT plasmid or mutant derivatives were supplied with 68 mg/l of chloramphenicol.

Triple site-saturating mutagenesis

Two degenerate primers were designed, one for the random mutagenesis of amino acid residues αR160 and αF161

(5′ gtg ggc act atg gcc aac nng/c nng/c tcg gac gcc aat agc gaa atc g 3′) and one for position βF24 (5′ ggc ggg gtt cca cca gcc c/gnn ctg cgg gcc gtt taa cag 3′). For the easy identification of real mutants, primer αR160X/αF161X was set up to remove a unique *Nco*I restriction site and primer βF24X to introduce an additional *Mse*I site, both leading to characteristic differences in the restriction patterns of wild-type and mutant plasmids (restriction sites underlined, mutations in bold). To reduce codon bias, the third position of each random codon was fixed to be a guanine or a cytosine. An outline of the used mutagenesis procedure is shown in Figure 2. In the first step, a 500 bp fragment containing all three randomized codons was PCR amplified with the two degenerate primers described above. The reaction was carried out with *Pfu Turbo* polymerase (Stratagene) under standard conditions, using 50 ng of pPAS2CAT as the template and 125 ng of each of the primers. The amplified fragment (megaprimer) was purified on gel with a QIAEX II gel extraction kit (Qiagen) and used as a forward and reverse primer set in a normal QuikChange site-directed mutagenesis procedure (Stratagene) according to the instructions of the manufacturer. After ethanol precipitation, the reaction mixture was resuspended in water and mutant plasmids were transferred to *E.coli* TOP10 by electroporation. Transformants were plated on LB agar (Sambrook *et al*., 1989). Mutant strains obtained by this procedure usually carry two plasmid populations with different mutations, one originating from each of the different strands of the original (chimeric) mutant plasmid. To solve this problem, all colonies were washed from the agar plates and used for plasmid preparation with a High Pure Plasmid Isolation Kit (Roche). The plasmid mix was then retransferred to *E.coli* to produce transformants, each carrying only one type of plasmid. The resulting amplified gene bank was then subjected to growth selection and screening. By enzymatic digestion of 20 single clones with *Nco*I and *Mse*I, the library was shown to contain >95% of triple mutants. Interesting mutants were sequenced with at least two times coverage of each base by BaseClear Holding (Leiden, The Netherlands). All sequences obtained were different.

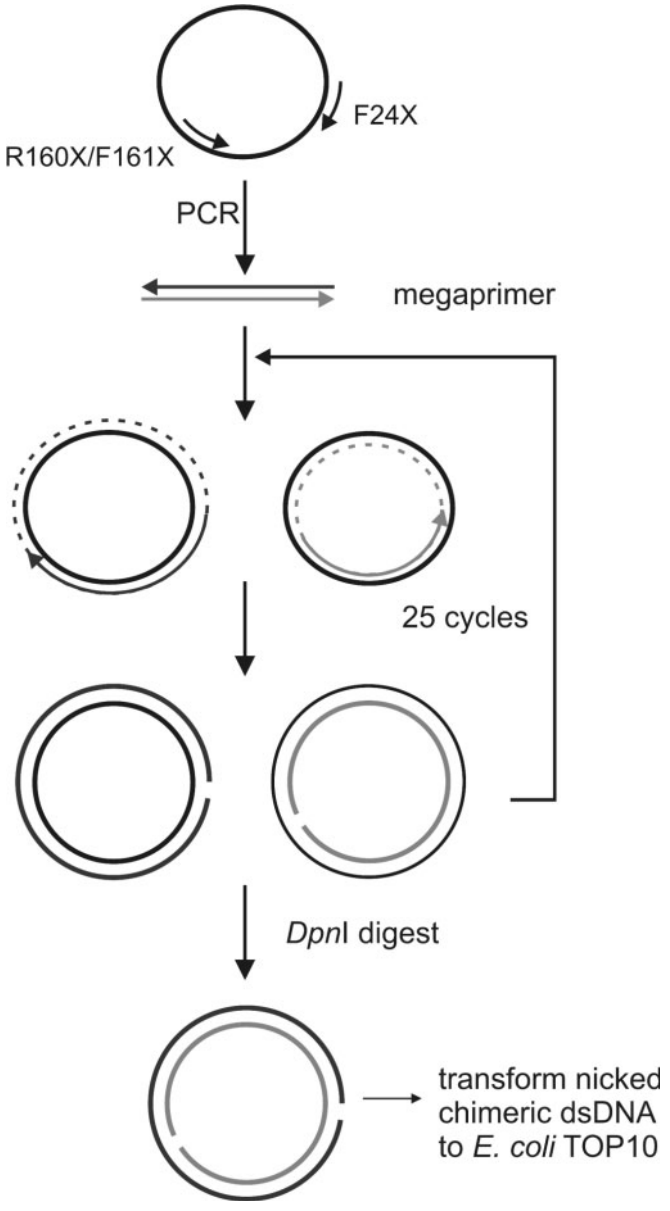

Fig. 2. Mutagenesis procedure used in this study. In the first step, a PCR product is prepared, using two degenerate primers that carry the desired randomized codons and the wild-type plasmid as the template. This product is then used as a megaprimer (three randomized positions) in a standard QuikChange site-directed mutagenesis procedure (Stratagene) to yield the desired triple mutants.

Selection of active mutants and screening for improved synthesis

Active mutants were selected by their ability to grow on phenylacetyl-L-leucine (PAL) as the sole source of leucine (Forney and Wong, 1989) in a minimal medium supplied with 0.2% (w/v) glucose and 15 g/l agarose as described previously (Gabor *et al*., 2004a). PAL was added at a concentration of 10 mg/l and 0.2% (w/v) arabinose wase included in the medium to induce protein expression from P_{BAD}. After 5 days of growth at 30°C, single colonies were transferred from the agar plates to 96-well microtiter plates (MTPs) filled with 200 µl of liquid LB medium per well. After overnight incubation at 30°C and 200 r.p.m. orbital shaking, 50 µl of 50% glycerol were added per well and plates were stored as frozen stocks at −80°C.

For the first round of screening by high-performance liquid chromatography (HPLC), plates were defrosted and 20 µl aliquots of the stock cell suspensions were transferred to fresh MTPs and used to inoculate 180 µl of LB medium. MTPs were incubated at 17°C with orbital shaking at 200 r.p.m. for 48 h, then 50 µl of 10% arabinose solution were added. An arabinose concentration of 2% in the culture medium resulted in the highest level of protein expression as established in preliminary experiments with the wild-type strain. After incubation for a further 24 h at 17°C, cells were collected by centrifugation for 5 min at 2500 r.p.m. in an MSE Mistral 2000 MTP centrifuge. They were resuspended in 200 µl of substrate solution per well (15 mM PGA and 10 mM 6-APA in 50 mM potassium phosphate buffer, pH 7.0) and incubated at 30°C. After 24 h, 10 µl of the reaction mixtures were transferred to fresh MTPs and quenched by the addition of 90 µl of HPLC eluent [340 mg/l sodium dodecyl sulfate and 680 mg/l $KH_2PO_4 \cdot 3H_2O$ in 30:70 (v/v) acetonitrile–water of pH 3.0 (adjusted with dilute phosphoric acid)]. Liquid handling was performed with a Plato 3001 automated pipetting station (Rosys, Switzerland) that also served as a 96-well MTP format autosampler for HPLC injection. HPLC analyses were carried out using a 3 cm Alltech Alltime C18 3u column in connection with Jasco PU-980 pumps and a Jasco MD-910 detector set at 214 nm. Compounds

were isocratically eluted at a flow rate of 5 ml/min with HPLC eluent, which allowed the analysis of one sample in 3 min. The ratio of the areas of the product peaks, i.e. ampicillin and PG, in each chromatogram was determined and compared with that of the *E.coli* (pPAS2CAT) control. Mutant strains exhibiting at least a 2-fold increased ampicillin/PG peak ratio or showing exceptionally high conversion rates were analyzed in more detail for their performance in the synthesis of ampicillin.

In this second round of HPLC screening, complete progress curves for the synthesis of ampicillin from 15 mM PGA and 25 mM 6-APA in 50 mM potassium phosphate buffer (pH 7.0) were recorded at 30°C, using periplasmatic extracts and enzyme concentrations of 5–10 μM. Extracts were prepared and analyzed for their content of active penicillin acylase as described in the next paragraph. A similar HPLC setup as for the robotic screening served for this analysis, but based on a 10 cm Chrompack C18 column. Here, compounds were eluted isocratically at a flow rate of 1 ml/min. Peak areas were related to the concentration of the respective compounds using calibration curves that were established with solutions of the pure compounds.

Preparation of periplasmatic extracts and enzyme purification

Periplasmatic extracts were prepared at different scales. For the verification of whole cell screening results in the second round of HPLC screening, 5 ml cultures were used as a starting material, while 1 l cultures were used for protein purification. In both cases, clones containing the wild-type or mutant PAS2 gene were grown in LB at 17°C with rotary shaking at 200 r.p.m. After 2 days of growth, the medium was supplied with 0.8% arabinose for the induction of protein expression. After a further 24 h of incubation, cells were harvested by centrifugation at 5000 *g* for 10 min (4°C). To prepare a periplasmatic extract, cells were resuspended in one-tenth of the original culture volume of ice-cold osmotic shock buffer (20% sucrose, 100 mM Tris–HCl, 10 mM EDTA; pH 8.0) and centrifuged as described above. Cell walls were disrupted by resuspending the cell pellet in one-fiftieth of the original culture volume of ice-cold 1 mM EDTA and cell debris was removed by another centrifugation step.

Wild-type PAS2 and mutant enzymes were purified as described previously (Gabor *et al*., 2004b). The amount of active enzyme in penicillin acylase preparations was determined by titration with the irreversible inhibitor phenylmethylsulfonyl fluoride (PMSF) according to Alkema *et al*. (1999). Enzyme preparations were incubated with different concentrations of PMSF in 50 mM potassium phosphate buffer (pH 7.0) at room temperature for 15 min. Residual activity was measured with NIPAB or ampicillin as a substrate. The release of 5-amino-2-nitrobenzoic acid from NIPAB was followed spectrophotometrically at 405 nm ($\Delta\varepsilon_{405\ nm} = 9.09\ mM^{-1}\ cm^{-1}$) with a Perkin-Elmer Bio40 UV/VIS spectrometer, and hydrolysis of ampicillin was monitored by withdrawing samples from the reaction mixtures at different time points in the initial phase of conversion and analyzing them by HPLC (10 cm Chrompack C18 column).

Determination of kinetic parameters

All enzymatic conversions were carried out in 50 mM potassium phosphate buffer (pH 7.0) at 30°C. Steady-state kinetic parameters of the mutant enzymes for the hydrolysis of PGA, PGM, ampicillin and cephalexin were determined by monitoring the initial rates of substrate conversion at various substrate concentrations by HPLC (10 cm Chrompack C18 column). Product concentrations were determined at several times in order to obtain at least three data points in the initial phase of conversion. Steady-state parameters of PAS2 were determined as described previously (Gabor *et al*., 2004b).

Kinetically controlled enzymatic synthesis of ampicillin and cephalexin was carried out by mixing enzyme with solutions of activated acyl donor (PGA or PGM) and 6-APA or 7-ADCA, respectively. The initial concentration of acyl donor was 15 mM in all experiments, and the concentration of β-lactam acyl acceptor varied between 0.5 and 200 mM. All reactants were monitored in time by HPLC analysis (10 cm Chrompack C18 column) and initial rates of formation of the antibiotic (v_{Ps}) and the hydrolyzed acyl donor (v_{Ph}) were determined.

Results and discussion

Structure-inspired semi-random mutagenesis

Penicillin acylase PAS2 (accession No. AY573298) and *E.coli* PA (accession No. AAA24324) share 51.4% of their amino acid residues. This degree of sequence identity was sufficiently high (>25%) to obtain a homology model of PAS2 based on the known 3-D structure of the *E.coli* enzyme (Duggleby *et al*., 1995). The model provided by the Swiss-Model service (http://www.expasy.org/swissmod/SWISS-MODEL.html) did not reveal significant changes in the geometry of the active site and the spatial orientation of several amino acid residues that were shown to affect significantly the catalytic behavior of *E.coli* PA (Alkema *et al*., 2002a,b) perfectly matched between the two proteins. This agreement and the conservation in a number of different other PAs, such as of *Proteus rettgeri* (accession No. A56681), *Alcaligenes faecalis* (accession No. AAD11517) and *Kluyvera citrophila* (accession No. A26528), prompted us to chose the three residues αR160, αF161 and βF24 of PAS2 (corresponding to positions α145, α146 and β24 in *E.coli* PA) as targets for mutagenesis. Alkema *et al*. (2000) showed that residues αR145 and αF146 have an important impact on the affinity of the *E.coli* enzyme for phenylacetylated substrates and the positioning of substrates towards the catalytic serine in the active site. Via an induced fit mechanism, they participate in the formation of the β-lactam binding site. When 6-APA or derived antibiotics are bound, both residues interact with the β-lactam moiety: αR145 with the negatively charged carboxylate group via two bridging water molecules and αF146 by van der Waals interactions with the 2β-methyl group of the thiazolidine ring (Done *et al*., 1998; Brannigan *et al*., 2000). The phenyl ring of the side chain of αF146 also participates in the formation of the acyl donor binding pocket by interacting with the phenyl ring of the acyl compound. On the other side of the binding pocket, another phenylalanine is located, βF24, which also shows hydrophobic interaction with the acyl donor (Alkema *et al*., 2002a).

Although the importance of the three residues in *E.coli* PA—and most likely also in PAS2—is obvious, it is difficult to predict what effect a specific mutation will have on the catalytic performance of the enzyme in a kinetically controlled synthesis reaction, because both the activated acyl donor and the antibiotic can serve for the formation of the covalent acyl-enzyme intermediate (EAc). Therefore, optimization of e.g. the enzyme acylation reaction in order to increase the reactivity

with the acyl donor usually also results in improved hydrolysis of the antibiotic formed. Also, engineering the enzyme with respect to improved deacylation by the β-lactam nucleophile is not straightforward, since increased reactivity of the β-lactam nucleophile is often accompanied by an increased specificity also for the β-lactam antibiotic. In the end, only subtle changes in the geometry of the acyl-enzyme or the orientation and activation of the deacylating nucleophiles cause altered rates of product and byproduct formation and influence Ps_{max}. These changes are mostly difficult to rationalize, which is particularly true for PAS2, for which an experimental 3-D structure is not yet available. Therefore, we chose to randomize the respective amino acid residues rather than to follow a completely rational approach. By simultaneously changing residues at three different sites, we were able to create a gene bank of high sequence diversity, while focusing on mutants that are likely to show significantly altered catalytic performance.

The procedure used to create the triple mutant bank of PAS2 is illustrated in Figure 2. Since two of the target positions for randomization concern adjacent amino acid residues, we only needed to design two degenerate primers: one for αR160 and αF161 and another for residue βF24. With these two primers, a (double-stranded) megaprimer was prepared by simple PCR amplification that could subsequently be used in a standard QuikChange mutagenesis procedure. In this way, 6000 original clones were created that were stored as an amplified gene library and screened for improved ampicillin synthesis.

Screening for higher ampicillin yields

The development of a direct screening method for increased antibiotic yields, e.g. by HPLC analysis, is difficult owing to the temporary nature of product accumulation. After reaching a maximum (Ps_{max}), the product concentration decreases owing to the enzymatically catalyzed hydrolysis of the antibiotic. Earlier experiments showed that an increase in $(v_{Ps}/v_{Ph})_{ini}$ at fixed reaction conditions is often coupled to an increase in Ps_{max} and can therefore be used as an alternative screening criterion (Alkema *et al*., 2002a). This is in agreement with Equation 1, which shows that an increase in β_0 and/or $1/\gamma$ (reflected by an increased rate ratio of synthetic and hydrolytic product formation) results in a higher Ps_{max} if α remains unchanged or is even decreased. A problematic feature of this screening approach is the fact that improved mutants with respect to Ps_{max} might be missed if they possess a superior α parameter, but have a similar $(v_{Ps}/v_{Ph})_{ini}$ as the wild-type enzyme. In the same way, mutants might seem improved upon screening, while actually not yielding more product if they have an unfavorably high α, which causes preference for the product over the activated acyl donor during enzyme acylation. Despite these limitations, we chose to use a screening procedure based on the described principle because of its relatively high throughput capacity and refine the search by a second, more detailed round of analysis.

In the first round of HPLC screening, cell suspensions of mutant clones were analyzed in MTPs for the produced amount of Ps (ampicillin) and Ph (D-phenylglycine, PG) from D-phenylglycine amide (PGA) and 6-APA at a certain time. PGA was used as the acyl donor owing to its high chemical stability and, consequently, low background hydrolysis under the applied reaction conditions. The peak ratio of ampicillin/PG formed after 24 h was compared with that of a wild-type PAS2 culture. Since we could not assume all reaction mixtures to be in the initial phase of conversion and the peak ratio changes in the course of reaction, several wild-type cultures with different induction levels were prepared in order to obtain a set of chromatograms with varying degrees of conversion. Each mutant chromatogram was then compared with the wild-type profile that showed the most similar conversion level. With this method, one 96-well MTP could be analyzed in about 5 h, leading to a maximum throughput of four plates per day. In order to decrease the number of clones that needs to be screened, clones expressing an active PAS2 variant were preselected on agar plates containing D-phenylacetyl-L-leucine (PAL) as the only source of leucine. Since only 20% of all mutants were found to be active, the screening effort for synthetic activity was reduced by a factor of 5 by only analyzing mutants that could grow on PAL medium. A total of 700 of these clones were subjected to a first round of HPLC screening (Figure 3).

In preliminary experiments, we had compared the recovery of active clones by this growth selection method with the detection of activity in MTPs due to the hydrolysis of 2-nitro-5-[(phenylacetyl)amino] benzoic acid (NIPAB), which is a well-known colorimetric substrate for penicillin acylases (Kutzbach and Rauenbusch, 1974). While all clones able to hydrolyze NIPAB were also found to grow on PAL, about 15% of the clones utilizing PAL could not hydrolyze NIPAB, which made us decide to use PAL for the recovery of active clones. Interestingly, the six truly improved mutants isolated in this study (see below) could not hydrolyze NIPAB or only at extremely low rates, which would have compromised their isolation if a prescreening on NIPAB had been included.

Besides selection on PAL plates, we also isolated transformants that could grow on medium supplied with amides such as D-phenylglycine amide, α-methylphenylglycine or D-mandelamide (racemic mixture) as the only source of nitrogen. However, none of the 500 mutants selected on these media showed promising ampicillin/PG peak ratios or high activity when tested for ampicillin synthesis. From the PAL preselection, in contrast, 50 clones exhibited enhanced synthetic behavior. The recovery of improved mutants from PAL plates but not from nitrogen-limited media can be attributed to the fact that only traces of leucine need to be released by hydrolysis to sustain growth, while local concentrations of nitrogen are required to

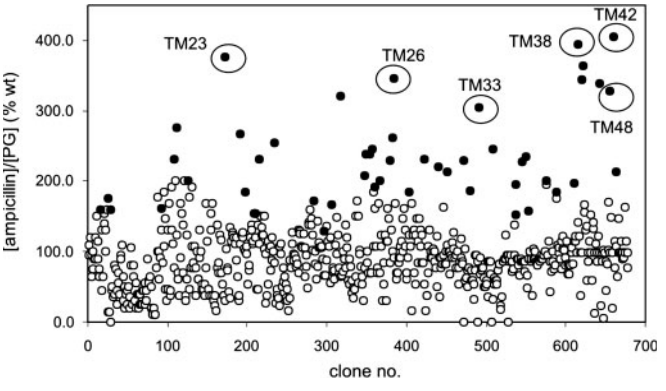

Fig. 3. Screening for improved PAS2 mutants by HPLC with whole cells. Clones represented by black dots were inspected in more detail in a second round of HPLC screening with periplasmatic extracts. Truly improved mutants that were subjected to further analysis are indicated by surrounding circles.

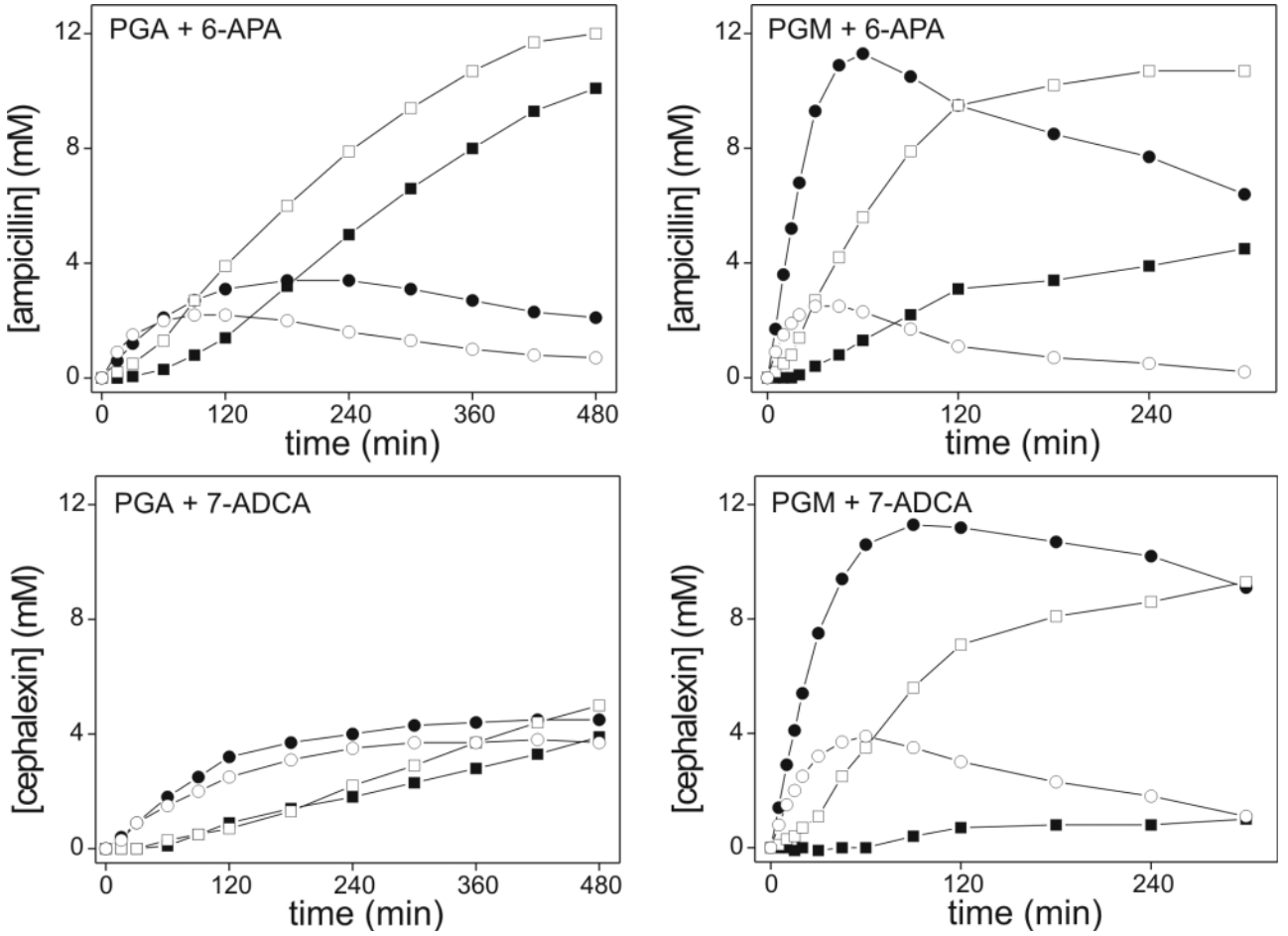

Fig. 4. Synthesis of ampicillin and cephalexin by wild-type PAS2 and TM23, using 15 mM acyl donor and 25 mM β-lactam nucleophile. When the amide PGA was used as the acyl donor, 75 nM wild-type and 7.5 μM mutant enzyme were used. With the ester PGM, 250 nM wild-type and 2.5 μM mutant enzyme were added. PAS2: open circles, antibiotic; open squares, PG. TM23: closed circles, antibiotic; closed squares, PG.

Table I. Amino acid residue changes (bold) of improved mutants of penicillin acylase PAS2

Name	Mutations
TM23	αR160**K** αF161**L** βF24**M**
TM26	αR160**Q** αF161**L** βF24**Q**
TM33	αR160R αF161F βF24**A**
TM38	αR160**P** αF161**A** βF24**V**
TM42	αR160**Q** αF161**R** βF24**I**
TM48	αR160**P** αF161**A** βF24**Q**

be in the μM range for the formation of reasonably sized colonies. Mutants that show improved synthetic performance but reduced hydrolytic activity may consequently not be able to grow on nitrogen-limited media.

The 50 improved mutants from the PAL preselection were tested in more detail in a second round of HPLC analysis. Here, complete progress curves for the kinetically controlled synthesis of ampicillin from PGA and 6-APA were recorded to reveal whether the respective mutant indeed allowed higher maximum product accumulation than the wild-type. Six of the mutants showed significantly improved synthetic performance and were subsequently also tested with another common β-lactam nucleophile, 7-ADCA, in the synthesis of cephalexin. Dependence on the type of acyl donor was revealed by using either PGA or the methyl ester derivative of D-phenylglycine, PGM, as the acyl moiety. Improvements were most pronounced for the synthesis of ampicillin for all mutants, particularly with PGM that allowed up to four times higher product accumulation than obtained with the wild-type enzyme. In general, the six mutants showed similar trends in the test reactions. Progress curves of triple mutant TM23 are shown as examples in Figure 4.

In contrast to the correspondence in synthetic behavior, the mutants were found to differ with respect to their amino acid residue substitutions (Table I). Remarkably, TM33 showed only one amino acid residue change, βF24A, corresponding to the βF24A *E.coli* PA single mutant that was reported to allow high antibiotic yields (Alkema *et al*., 2002a). However, improvements for the *E.coli* mutant were most pronounced in the synthesis of cephalexin, whereas TM33 performed best in the production of ampicillin, in both cases using PGM as the acyl donor. Although TM33 did not carry mutations at the other two sites, it was confirmed as a true triple mutant by restriction analysis and the presence of altered codons for residues αR160 and αF161. The other five mutants contained amino acid substitutions at all three target positions. Although some mutations, i.e. αR180K and αF181L, had earlier been found to influence antibiotic synthesis in *E.coli* PA single mutants

(Alkema *et al*., 2002a,b), the effects of most substitutions and their combination have not been studied.

Kinetic characterization of the best mutants

Whereas antibiotic yields were clearly improved for all mutants with PGA as the acyl donor (120–260%), synthesis rates were strongly decreased, being <1% of the wild-type rates. Activities with PGM were much higher for all mutants, but mutants TM26, TM42 and TM48 still maximally reached 3.6% of the PAS2 synthesis rates. For a detailed kinetic characterization, we therefore focused on the three other mutants, TM23, TM33 and TM38, that exhibited 10–20% of the wild-type activity in antibiotic formation. All experiments were carried out with purified enzyme preparations.

The steady-state parameters for the hydrolysis of the acyl donors used in this study and of the synthesized antibiotics are summarized in Table II. In agreement with the low conversion rates observed in the synthesis experiments, the k_{cat} values of all mutants for the acyl donors tested were decreased. TM23, TM33 and TM38 showed a clear preference for hydrolysis of the ester compared with the amide substrate. Surprisingly, the wild-type enzyme was slightly more specific for the amide AD, although enzyme-catalyzed ester hydrolysis is generally expected to be faster owing to the comparatively lower chemical stability of the ester bond (Polgar, 1989). Although several hypotheses exist that, for example, relate this unexpected feature to a distortion of the normally planar amide bond towards a structure that more resembles the tetrahedral transition state that leads to the formation of the covalent acyl-enzyme intermediate (James *et al*., 1980; Polgar, 1989; Hedstrom *et al*., 1992), no structural evidence for the presence of the proposed mechanisms in PA has yet been provided.

The increase in relative esterase/amidase activity of the mutants was also reflected in their α parameters for the synthesis of both ampicillin and cephalexin, which were much lower with PGM as the acyl donor than with PGA (Table III). Whereas with PGM the α values of all mutants were smaller than that of the wild-type enzyme, partly explaining the high antibiotic yields, α was up to 17-fold higher for the amide acyl donor. This seems to be in contradiction with the observed increase in antibiotic accumulation with PGA as the substrate. However, not only the competition between antibiotic and activated acyl donor for acylation of the enzyme determines Ps_{max}, but also the competition between the β-lactam nucleophile N and water for deacylation of the covalent acyl-enzyme intermediate (EAc), which is described by the parameters β_0 and $1/\gamma$. With 10–40-fold increases in β_0 and even up to 60 times higher values for $1/\gamma$ (Table III), improvements with respect to deacylation of EAc by the tested β-lactam compounds were large enough to compensate for the relative decrease of specificity for PGA compared with the antibiotic

Table II. Steady-state kinetic parameters of penicillin acylase PAS2 and the improved mutants

Enzyme	PGA			PGM		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
PAS2	25	12	2.1	24	14.3	1.7
TM23	0.7	122	0.006	2.0	13.4	0.15
TM33	0.13	29	0.0053	4.8	53.4	0.09
TM38	0.15	33	0.005	1.8	13.7	0.13
	Ampicillin			Cephalexin		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
PAS2	16	0.6	26.7	20	1.3	15.4
TM23	3.4	2.6	1.31	0.6	1.7	0.35
TM33	2.3	2.2	1.05	0.4	0.9	0.44
TM38	2.4	2.4	1.00	0.8	2.8	0.29

Table III. Complex kinetic parameters α, β_0 and γ of penicillin acylase PAS2 and improved mutants

Enzyme	Acyl donor	Ampicillin			Cephalexin		
		α	β_0 (mM^{-1})[a]	$1/\gamma$[b]	α	β_0 (mM^{-1})[a]	$1/\gamma$[b]
PAS2	PGA	13.2	0.5	6	7.3	0.5	59
	PGM	16.4	0.5	6	9.1	0.5	59
TM23	PGA	218	6.3	364	58.8	18.4	69
	PGM	8.8	6.3	364	2.4	18.4	69
TM33	PGA	209	10.2	286	88.8	21.5	154
	PGM	11.6	10.2	286	4.9	21.5	154
TM38	PGA	200	4.9	167	57.2	21.1	58
	PGM	7.6	4.9	167	2.2	21.1	58

[a]Mean of two independent experiments, using the amide PGA and the ester PGM as the acyl donor, respectively. Coefficient of variations were <12%.
[b]The parameter γ was obtained by fitting Ps vs Ph plots ([AD] = 15 mM, [N] = 25 mM) with Equation 1 and the experimentally determined values for the parameters α and β_0. The given value is the average obtained from analyzing two different curves, one with PGA and the other with PGM as the acyl donor. The coefficients of variation were <10% for all data. Average values are given for β_0 and $1/\gamma$, since these parameters are independent of the type of acyl donor used.

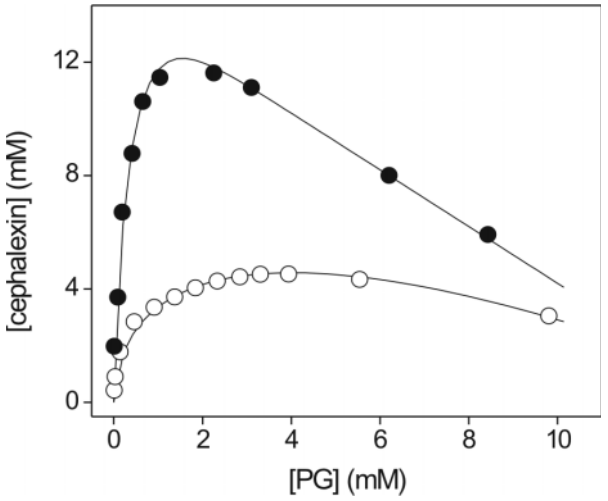

Fig. 5. Formation of synthesis product Ps (cephalexin) and hydrolytic byproduct Ph (PG) during conversion of 7-ADCA and the amide acyl donor PGA (open circles) or the ester PGM (closed circles) by TM38 penicillin acylase. Lines represent fits obtained with Equation 1, using α = 57.2 (PGA), α = 2.2 (PGM) and β_0 = 21.1 mM^{-1}. Values obtained for $1/\gamma$ were 56 (PGA) and 59 (PGM).

and allowed higher yields than with the wild-type enzyme. Since the formation of both reaction products, Ps and Ph, occurs via the same covalent intermediate (EAc), β_0 and $1/\gamma$ should be independent of the type of AD used, which is in agreement with our experimental results. Whereas for PAS2 $(v_{Ps}/v_{Ph})_{ini}$ could be determined for a whole range of concentrations of the β-lactam nucleophile (1–200 mM), allowing the experimental determination of both β_0 and $1/\gamma$ (Gabor *et al.*, 2004b), only β_0 could be measured for the three mutants. This is due to the fact that the synthetic reaction was so much preferred with these enzymes that already at relatively low nucleophile concentrations the amount of hydrolytic product was below the detection limit in the initial phase of conversion. Therefore, only β_0 could be measured at 6-APA and 7-ADCA concentrations below 5 mM, whereas $1/\gamma$ needed to be obtained by fitting [Ps] vs [Ph] plots that were derived from progress curves (Figure 4) with Equation 1, using the experimental values of α and β_0. Good agreement was observed between the data derived from curves based on the use of the amide and the ester acyl donor (Figure 5), supporting the assumption that the formation of the acyl-enzyme intermediate is the rate-limiting step of the conversion.

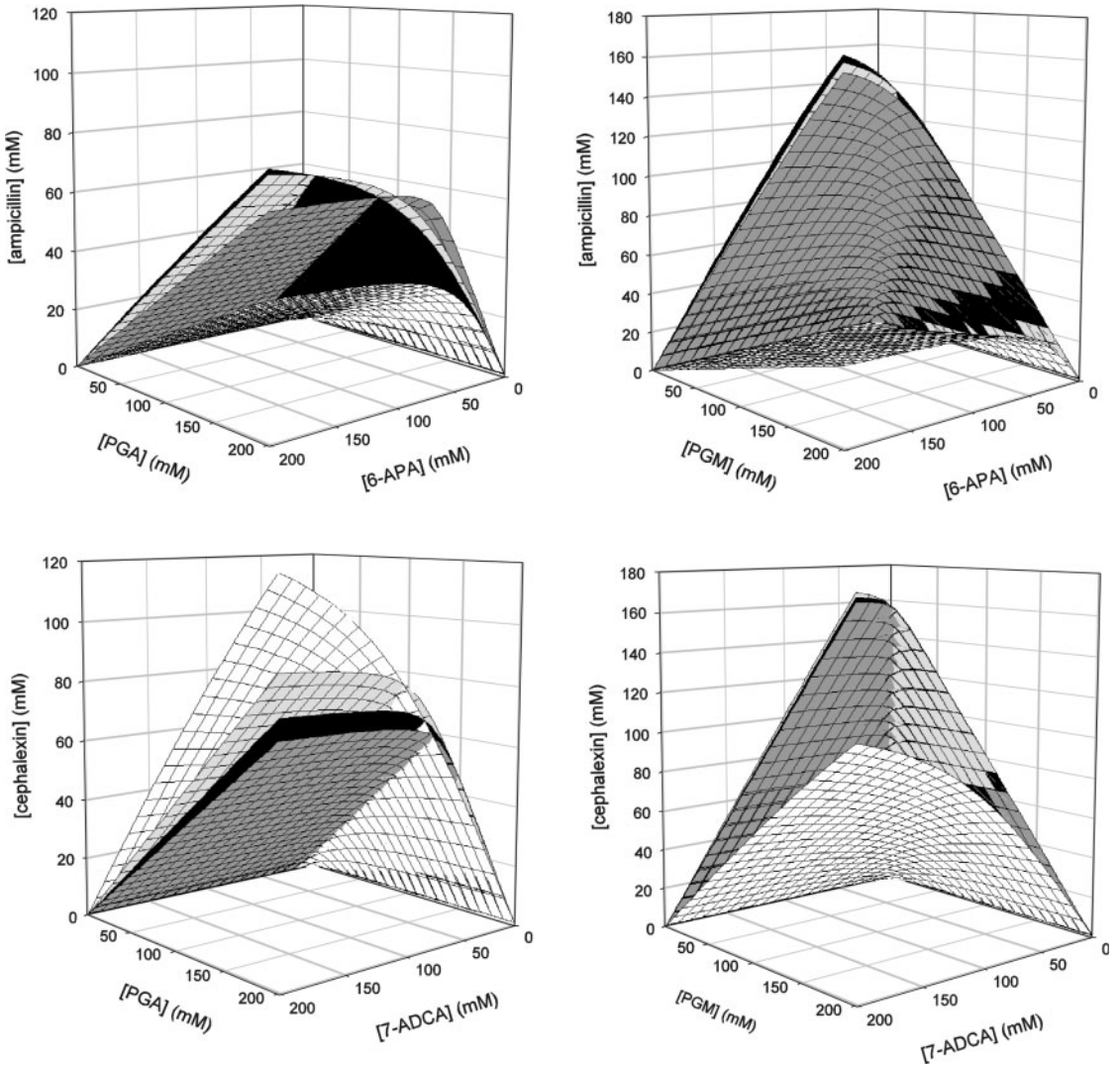

Fig. 6. Maximum accumulation of ampicillin and cephalexin in PA-catalyzed, kinetically controlled conversions, using different substrate concentrations. Data were simulated with Equation 1, using the complex kinetic parameters α, β_0 and γ given in Table III. PAS2 (white); TM23 (black); TM33 (light gray); and TM38 (dark gray).

Interestingly, when 6-APA was used as the β-lactam nucleophile, improvements were more pronounced for $1/\gamma$ than for β_0, whereas with 7-ADCA the opposite situation occurred. For mutants TM33 and 38, virtually no increase in $1/\gamma$ was observed in the synthesis of cephalexin. The high values of $(v_{Ps}/v_{Ph})_{ini}$ at low concentrations of 7-ADCA indicate that the affinity for this β-lactam nucleophile became higher owing to the mutations in the substrate binding pocket, while the exclusion of water from the active site at high 7-ADCA concentrations was obviously not improved. In contrast, binding of 6-APA in the active site seemed to suppress greatly the nucleophilic attack of water on the acyl-enzyme intermediate.

Antibiotic yields at high substrate concentrations

In addition to by α, β_0 and γ, the maximum product yield of a given reaction is also determined by the initial substrate concentrations $[N]_0$ and $[AD]_0$ and can, in homogeneous systems, be calculated with Equation 1 as shown by Youshko and Svedas (2000). To predict the performance of TM23, TM33 and TM38 at substrate concentrations higher than the experimentally used levels, we modeled the maximum antibiotic accumulation for a range of substrate concentrations in the homogeneous regime, using the parameters given in Table III.

From Figure 6, it is apparent that all three mutants have a higher potential for the synthesis of antibiotics derived from 6-APA, particularly ampicillin, than from 7-ADCA. Although higher yields of cephalexin are predicted for low concentrations of 7-ADCA with PGA as the acyl donor, which is in agreement with our experiments (Figure 4), wild-type PAS2 allows significantly higher yields at nucleophile concentrations above 50 mM with about 100-fold higher conversion rates. When PGM and 6-APA are used, the drawback of lower activity (10–20% of wild-type) may, however, become unimportant in view of the 4–5 times higher ampicillin concentrations that can be reached over the whole range of substrate concentrations. This is particularly interesting since the reaction can be accelerated just by adding more enzyme, while the maximum level of product accumulation that is reached in a kinetically controlled synthesis reaction can only be increased by using a better enzyme. The unproductive loss of activated acyl donor is also drastically reduced when using the mutant enzymes: at 200 mM 6-APA concentration, about 80% of PGM is converted to ampicillin with the mutant enzymes, whereas only 17% is used for synthesis with the wild-type PAS2 penicillin acylase, the remainder being hydrolyzed to D-phenylglycine (Figure 6).

In conclusion, our study demonstrates that focusing random mutagenesis to amino acid residues that are expected to affect the catalytic behavior of the target enzyme based on sequence and structural information is an efficient tool for increasing the frequency of biocatalysts with a desired property in a mutant library. With this method, three mutant penicillin acylases carrying different amino acid substitutions were recovered that allow significantly higher ampicillin yields with PGM than the wild-type enzyme, while retaining relatively high catalytic activity.

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